

**METHOD FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE****AND OTHER PRION RELATED DISORDERS****5 CROSS REFERENCE TO RELATED APPLICATION**

This application claims priority from U. S. Provisional Application No. 60/263,841 filed on January 23, 2001 and which is hereby incorporated by reference.

**FIELD OF THE INVENTION**

10 This invention relates to a method for diagnosing Alzheimer's Disease and other prion related disorders in patients using multiple biomarkers. More specifically, the present invention provides a method for monitoring the levels of glycoproteins in patients suffering from such disorders by using wheat germ agglutinin as a biomarker to specifically bind to glycoproteins isolated from cerebrospinal fluid (CSF). In addition, this method combines the measurements of wheat germ agglutinin-reactive glycoproteins in CSF with alteration in glycosylation patterns in such patients.

**BACKGROUND OF THE INVENTION**

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20 Presently, the only diagnosis of a number of prion related disorders including transmissible spongiform encephalopathies (TSE) and dementias found, e.g., in Alzheimer's Disease (AD) is neuropsychological assessment by *post mortem* examination of the brain. A common problem in interpreting *ante mortem* cerebrospinal fluid (CSF) results has been the uncertainty of clinical diagnosis. It has been estimated that the accuracy of diagnosis of AD by clinical examination is only about 80-90% at best (Growdonk 1999). Therefore, some individuals diagnosed as AD may be suffering from a non-AD dementia and some of the non-

AD samples may be from individuals with latent AD. Therefore, there is a need to identify specific biochemical markers of AD and other dementias and prion related disorders .

Studies have shown that the level of tau (Ara et al., 1995; Tojanowski et al., 1996) and A $\beta$  (Nakamura et al., 1994; Motter et al., 1995; Galasko et al., 1998; Kani et al., 1998) are altered in AD CSF. Several studies have demonstrated that the use of multiple biomarkers in combination may improve diagnosis. (Galasko et al., 1998; Kanai et al., 1998). However, the present biomarkers do not provide the sensitivity or specificity for AD diagnosis (Working Group et al., 1998).

The glycosylation patterns of many proteins are altered in severe disease states (Lis & Sharon, 1993) including Alzheimer's Disease (AD). The lectin blotting technique has been used to examine the glycosylation of proteins in non-AD and AD CSF (Savage et al., 1992). Alterations in the glycosylation pattern of several proteins in the AD brain have been studied (Guevara et al., 1998; McFarlane et al., 1999). Previous studies demonstrated that the level of an unusual glycoform of acetylcholinesterase (AChE) is increased in the AD brain and CSF (Saez-Valero et al., 1993, 1997). This glycoform can be distinguished from other AChE isoforms because this unusual isoform does not bind to the lectin, *Conavalia ensiformis* (ConA). However, until the present invention, changes in AChE expression and glycoform distribution have not been found to be of sufficient sensitivity or specificity to be useful diagnostic markers for AD other dementias.

## **SUMMARY OF INVENTION**

The present invention relates to a method for diagnosing transmissible spongiform encephalopathies (TSE) such as Mad Cow disease, Scrapie and Kuru, dementias such as

Alzheimer's Disease, and other prion related disorders by measuring the level of glycoproteins isolated from fluid from subjects with the above conditions that bind to wheat germ agglutinin (WGA).

In addition, the present invention provides a method for increasing the sensitivity for  
5 diagnosing transmissible spongiform encephalopathies (TSE) such as Mad Cow disease, Scrapie and Kuru, dementias such as Alzheimer's Disease, and other prion related disorders by combining the use of measuring the levels of glycoproteins bound to wheat germ agglutinin with the assay for measuring the binding of AChE isoform with ConA.

The present invention further provides a method for increasing the sensitivity for  
10 diagnosing transmissible spongiform encephalopathies (TSE) such as Mad Cow disease, Scrapie and Kuru, dementias such as Alzheimer's Disease, and other prion related disorders by combining the use of measuring the levels of glycoproteins bound to wheat germ agglutinin with the assay for measuring the binding of butyrylcholinesterase with ConA.

### **DETAILED DESCRIPTION OF THE FIGURES**

**Figure 1** shows the densitometric profile of the staining pattern after analysis of a typical AD CSF sample and a non-AD CSF sample by SDS-PAGE. Arrows indicate major WGA-reactive glycoproteins, with apparent molecular weights of 58 K, 90 K and 100 K, which are decreased in the AD samples.

**Figure 2** shows quantitative analysis of three WGA -reactive glycoproteins (58 K, 90 K and 100 K) in non-AD (o) and AD (●) CSF samples. The intensity of all three biotinylated protein bands was significantly lower in AD CSF than in controls ( $P < 0.001$ , Student's  $t$  test). Mean density

values  $\pm$  SEM for the three WGA-reactive glycoproteins were non-AD: 58 K  $82 \pm 4$ , 90 K  $91 \pm 4$ , 100 K  $82 \pm 3$ , AD: 58 K  $42 \pm 6$ , 90 K  $53 \pm 7$ , 100 K  $44 \pm 6$ . Mean value was calculated (WGA staining density of 58 K band + WGA staining density of 90 K band + WGA staining density of 100 K band / 3). The value of W in AD CSF was approximately 50 % lower than controls.

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**Figure 3** shows a plot of the sum of the mean densities for WGA staining of three WGA - reactive glycoproteins (W) versus % AchE unbound to *Conavalia ensiformis* (ConA) in AD(●) and non- AD (○) *ante mortem* CSF samples. Dotted lines show cut-off values which discriminates between probable AD and non-AD samples.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the diagnosis of transmissible spongiform encephalopathies (TSE) such as Mad Cow disease, Scrapie and Kuru, dementias such as Alzheimer's Disease, and other prion related disorders in a patient comprising the steps of providing a sample of an appropriate body fluid from the patient, isolating proteins from the sample, labeling WGA with biotin or any convenient label, mixing the sample with WGA, determining the level of WGA binding and comparing the level of WGA binding to a known standard.

In a preferred embodiment, the relative amount of proteins that bind to WGA is measured. Measurement of the reactive bound WGA-protein may be carried out in any convenient manner for example, by using biochemical analysis such as HPLC, mass spectrometry or immunological techniques such as ELISA, or assays. However, a particularly preferred means of measuring the WGA-reactive proteins involves a lectin-binding analysis.

The body fluid analyzed can be cerebrospinal fluid (CSF), blood or blood plasma.

1 In a preferred embodiment, the method of the present invention is used for diagnosis of  
Alzheimer's Disease. The present inventors demonstrated that the levels of glycosylation  
patterns which bound to the WGA were decreased in patients with AD CSF compared to non-  
AD controls. More importantly, the glycosylation patterns of CSF proteins that bound with  
5 other lectins did not differ between non-AD and AD samples. Therefore, in a preferred  
embodiment, the present invention is used to diagnosis Alzheimer's Disease.

The present invention further provides a method for increasing the sensitivity and  
specificity for diagnosis of dementias and prion disease by using multiple biomarkers. In  
particular, in another embodiment, the level of WGA binding to glycoproteins isolated from  
10 samples from subjects manifesting conditions of dementia or prion disease is compared to the  
percentage of acetylcholinesterase (AChE) unbound to ConA.

Previous AD studies have revealed an increase in an unusual glycosylated isoform of a  
AChE in AD CSF that does not bind ConA. Therefore, in a preferred embodiment, the method  
of comparing the level of WGA binding to glycoproteins isolated from samples from subjects  
15 manifesting conditions of dementia or TSE to the percentage of AChE unbound to ConA to  
increase sensitivity and specificity is used to diagnose Alzheimer's Disease.

In still another embodiment, the present invention provides a method for increasing the  
sensitivity and specificity for diagnosis of transmissible spongiform encephalopathies (TSE)  
such as Mad Cow disease, Scrapie and Kuru, dementias such as Alzheimer's Disease, and other  
20 prion related disorders by comparing the level of WGA binding to glycoproteins isolated from  
samples from subjects manifesting conditions of dementia or TSE to the percentage of  
butyrylcholinesterase (BuChE) unbound to ConA.

It has been established that approximately 93.6% of the BuChE in CSF of AD subjects binds ConA. Accordingly, in a preferred embodiment, the method of comparing the level of WGA binding to glycoproteins isolated from samples from subjects manifesting conditions of dementia or prion disease to the percentage of BuChE unbound to ConA to increase sensitivity and specificity is used to diagnosis Alzheimer's Disease.

While evidence thus far does not show alterations in levels of WGA reactive glycoproteins *per se* in Parkinson's Disease or multiple sclerosis, the method of the present invention may nonetheless prove useful in the diagnosis of these disorders as well.

### **EXAMPLES**

#### **Materials and Methods**

##### **I. Collection of CSF samples**

CSF samples from 21 non-AD (5 non-neurological diseases, 5 epilepsy, 5 hydrocephalus, 3 polyneuropathies and 3 intracranial hypertension: mean age  $71 \pm 3$  years) and 11 probable AD (mean age  $62 \pm 6$  years) cases were collected from patients at the Hospital Universitario San Carlos of Madrid. Patients were diagnosed for probable AD according to NINCDS-ADRDA criteria (McKhann *et al.*, 1984). The duration of disease from the Alzheimer's Disease group was [mean (SEM)] 1.9 (0.3) years and the average clinical dementia rating (CDR) was 1.4 (0.18). Lumbar punctures were performed in lateral decubitus and the CSF was stored immediately at  $-40^{\circ}\text{C}$ . No drugs were taken 12 hours before the collection of CSF.

None of the patients received anti-cholinesterase therapy.

There was no significant difference in total protein concentration in non-AD CSF or AD CSF (non-AD CSF  $1.05 \text{ mg/mL} \pm 0.08$ , AD CSF  $0.96 \text{ mg/mL} \pm 0.12$ ,  $P = 0.546$ ) as determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

## II. Lectin blotting

CSF was mixed with an equal volume of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 1 M Tris-HCl (pH 6.8), 4%(w/v)SDS, 2%(w/v) glycine, 0.01% (w/v) bromophenol blue and 10%(v/v)  $\beta$ -mercaptoethanol and heated in a boiling water bath for five minutes. Aliquots (15  $\mu$ L) of this mixture were applied to 7.5% SDS-PAGE. After electrophoresis proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at a constant current of 300 mA overnight. Membranes were blocked in 0.5% hydrolyzed casein in phosphate-buffered saline (PBS), pH 7.4, for 1 h. The blocked membranes were then incubated with 12.5  $\mu$ g/mL biotin-labeled lectins, *Triticum vulgaris* agglutinin (wheat germ; WGA), *Conavalia ensiformis* agglutinin (ConA), *Ricinus communis* agglutinin (RCA<sub>120</sub>) or *Lens culinaris* agglutinin (LCA) (Sigma-Aldrich Pty. Ltd., Seven Hills, Australia) in Tris-buffered saline-Tween20 (polyoxyethelyene-sorbiton monolaurate) (T BST) for 1 h at room temperature. After washing the membranes with TBST, glycoproteins were visualized using (1:1000 dilution) NeutrAvidin-conjugated to alkaline phosphatase (Pierce Chemicals Co., Rockford, Illinois, USA) and developed with a mixture of Fast Red (6 mg/ml in AP buffer) and naphthol AS-MX substrate (0.4 mg/ml in distilled water). The stained blots were scanned using a UMAX Astra 2400S scanner and bands from lectin blots were quantified by densitometry using the NIH Image (Version 1.57) program written by W. Rasband (National Institutes of Health, Bethesda, MD). Peak areas were defined as the area under the curve between two minima after subtracting a background value obtained from a blank region of the blot.

**Example I Wheat Germ Agglutinin-Reactive glycoproteins (WGA binding index)**

The staining of CSF proteins with biotinylated WGA was found to be less intense in the AD samples than in non-AD controls. However, the staining of CSF proteins with other lectins (ConA, LCA or RCA) did not differ between non-AD and AD samples (see, Fig. 1).

5 Several WGA-reactive proteins were decreased in AD CSF. Three of the most intensively stained glycoproteins, with apparent molecular weights of 58 K, 90 K and 100 K, were chosen for quantitative analysis (see, Fig. 2). The staining of all three glycoproteins with WGA was significantly decreased ( $P < 0.001$  as assessed by the Student's t test) in AD CSF compared to controls (see, Fig. 2). The percentage decrease in AD CSF was approximately 30 %, 40 % and 45 % for the 58 K, 90 K and 100 K proteins, respectively. As all three WGA glycoproteins were decreased in AD CSF, a mean value ( $W$ ) for the density of all three bands was calculated (WGA staining density of 58 K band + WGA staining density of 90 K band + WGA staining density of 100 K band / 3)(see, Fig. 2). The value of  $W$  in AD CSF was approximately 50 % lower than controls.

**Example II Use of Multiple AD Biomarkers**

To increase the sensitivity and specificity of the present method for diagnosis of AD, the use of multiple biomarkers was explored by combining the measurements of AChE glycosylation with the WGA binding index. The measurements of the percentage of AChE unbound to ConA (an index of the unusual AChE isoform) were plotted against the staining intensity of the sum of the mean densities (mean density values of 58 K + 90 K + 100 K) of the three proteins detected by biotinylated WGA (see, Fig. 3). The majority of non-AD samples were situated in the bottom right hand corner of the plot. The sensitivity and specificity of the combined measurements were



calculated by drawing a dotted line separating the non-AD group and AD group (see Fig. 3).

The sensitivity of the test was defined by the number of non-AD measurements in the AD boundary and the specificity was defined as the number of AD measurements in the non- AD boundary. The combined measurements of the alterations in the glycoproteins (WGA-reactive index and AChE isoform index) was 82% and 86% detection for sensitivity and specificity, respectively.

This application incorporates by reference the following publications:

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